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The effect of temperature changes on *in vitro* slow wave activity in the equine ileum

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Keywords: horse; ileus; slow waves; intestine; temperature

Summary

Reasons for performing study: Slow waves are rhythmic pacemaker currents generated by the gastrointestinal pacemaker cells, the interstitial cells of Cajal, and represent the rate-limiting step for small intestinal smooth muscle contractions. Therefore, factors that affect slow wave activity may also influence contractile activity. It is not known how temperature changes may influence slow wave activity in the horse. This could be of relevance during colic surgery if cooling of exposed intestine resulted in reduced slow wave activity potentially exacerbating post-operative ileus.

Objectives: To evaluate the effect of temperature changes on *in vitro* slow wave activity of normal equine ileum using intracellular recording techniques.

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Study Design: *In vitro* experimental study.

Methods: A segment of ileum was collected immediately following euthanasia from 9 horses, euthanased for reasons unrelated to the gastrointestinal tract. Intracellular recordings of membrane potentials were made from individual smooth muscle cells. The temperature of the tissue bath was altered during the course of each experiment across a range of 27-41°C. All data were recorded and stored using a computer-interfaced acquisition system. A software package was used to analyse slow waves frequency, duration, amplitude and resting membrane potential.

Results: In all 9 horses, slow wave frequency was highly temperature sensitive and approximately linearly related to the temperature over the range studied, increasing by 0.5 cycles/min for each one degree increase in temperature ($p < 0.001$). The initial slow wave frequency resumed when the temperature was returned to 37°C. The recovery time appeared to be directly related to the duration for which the temperature had been changed.

Conclusions: Slow wave frequency in the equine ileum is highly temperature sensitive. As post-operative ileus is a major cause of morbidity and mortality in the horse, the negative effect of lower temperatures on slow wave, and therefore contractile activity, should be considered.

Introduction

Phasic contractions of intestinal smooth muscle cells are initiated and timed by pacemaker currents generated by the interstitial cells of Cajal (ICC) [1-4]. These continuously fluctuating currents are conducted to smooth muscle cells through low resistance electrical junctions [5, 6] where they evoke similar membrane potential fluctuations (slow waves) [7]. If the slow wave current exceeds an electrical threshold, voltage-dependent, dihydropyridine-sensitive

(L-type) Ca^{2+} channels are activated resulting in calcium influx, an action potential and consequent contraction of smooth muscle cells [8]. Various inputs, including neuronal, mechanical and endocrine factors can condition the smooth muscle cell response to the ICC currents to either facilitate or reduce the possibility of the electrical threshold for Ca^{2+} channel activation being reached [8]. However, it is the frequency of slow waves that will determine the potential rate of intestinal smooth muscle contraction [4]. Factors that affect this activity may therefore also influence the contractile activity of the intestinal tract.

The effect of temperature alterations on small intestinal *in vitro* slow wave activity has previously been evaluated in laboratory animals [9-11]. It was concluded that this activity is highly temperature-dependent, possibly due to reliance of cellular metabolic processes for its generation [11]. The activity of membrane channels regulating ionic conductances and thereby also membrane potential [12] may be one such process.

Hudson *et al.* [13] previously demonstrated and characterised slow wave activity in the equine ileum using *in vitro* intracellular recording techniques under normal physiological temperature conditions. However, the effect of temperature changes on this electrical activity has not been previously assessed in the horse. This may be of some clinical relevance during surgery where often lengthy general anaesthesia may result in mild to moderate hypothermia of the horse [14-15]. This may be further exacerbated if large volumes of intravenous fluids of ambient temperature are administered concurrently [14]. Furthermore, separate studies in experimental animals demonstrated that a significant amount of heat could be lost by evaporation of moisture from exposed intestinal segments during open abdominal surgery [16-17]. If this cooling has a negative effect on slow wave activity, and consequent contractile activity, this may increase the risk of the patient developing post-operative ileus.

75

76 Post-operative ileus (POI) is a significant cause of morbidity and mortality in the horse [18-
77 20] and although several risk factors have been identified [21-23], a limited understanding of
78 the underlying pathophysiological processes still exists. This includes the direct association
79 between hypothermia and the development of POI. Although an association between duration
80 of anaesthesia (and therefore an increased risk of developing hypothermia) [14-15] and the
81 development of POI has been demonstrated [21-23] definitive documentation of intestinal
82 cooling during exploratory abdominal exposure in the horse is lacking. Nevertheless, it seems
83 reasonable to assume that this may also occur in the horse as has been previously
84 demonstrated in experimental animals (rabbits and piglets) [16-17].

85

86 The purpose of this study was therefore to test the hypothesis that slow wave frequency would
87 be influenced by tissue temperature also in the horse. Furthermore, any changes on slow wave
88 morphology that may occur during this process would be evaluated.

89

90

91 **Materials and Methods**

92 A segment of ileum (level with the midpoint of the ileo-caecal fold) was collected
93 immediately following euthanasia by intravenous administration of pentobarbitone^a from 9
94 adult horses of mixed breeds. The median age of the horses was 6 years, the mean age 8.2
95 years (range 1-18 years) and comprised 5 geldings, 2 mares and 2 entire males. All horses
96 were euthanased for reasons unrelated to the gastrointestinal tract.

97 Following collection, tissue samples were immediately placed in modified oxygenated Krebs
98 solution at room temperature (118 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl₂, 1.2 mM
99 MgSO₄, 1.15 mM NaH₂PO₄, 25 mM NaHCO₃ and 11.1 mM glucose). A one millimetre

100 thick section was cut from the ileum using a double-bladed knife in an orientation parallel to
101 the outer longitudinal muscle layer. The section was pinned on Sylgard^b in a tissue bath, and
102 following removal of the mucosal layer, superfused with warmed, oxygenated (95% O₂, 5%
103 CO₂) Krebs solution to ensure that the temperature in the tissue bath was kept between 37.0-
104 37.5°C during an initial 60 min equilibration period. The time taken from tissue collection to
105 the start of this equilibration period would range from 10-20 min. The temperature of the
106 tissue bath was continuously monitored and recorded using a temperature probe^c that was kept
107 in the bath throughout all experiments.

108
109 Intracellular recordings were made from smooth muscle cells using glass microelectrodes
110 filled with filtered 2M potassium chloride and with resistances ranging from 20-45MΩ. A
111 calibrating graticule in the eyepiece of the dissecting microscope was used to determine the
112 exact position of impaled smooth muscle cells relative to the thickness of the muscle layer.
113 All recordings were made in the longitudinal muscle layer close to the myenteric plexus
114 region (within 20% of the longitudinal muscle layer). Cellular recordings were considered
115 acceptable if there was a sharp initial drop in voltage upon cellular impalement and if the
116 resting membrane potential (RMP) remained stable thereafter. The RMP was measured at the
117 most negative reading for each impalement.

118
119 In order to facilitate stable cellular impalements, the L-type Ca²⁺ channel blocker nifedipine^d
120 (1 μM) was added to the superfusion fluid prior to commencing recording in all experiments
121 and was maintained throughout.

122
123 The sodium channel blocker tetrodotoxin^d (TTX) (1 μM) was added to the superfusion fluid in
124 2 experiments in order to establish that the recorded activity was non-neuronal in origin.

125

126 Once the equilibration period was completed and recordings commenced, the temperature in
127 the tissue bath was gradually altered covering a temperature range of 27-41°C. The
128 temperature was altered by adjusting the thermostat of the water bath used to warm the Krebs
129 solution prior to it reaching the tissue bath. Typically this would involve gradually increasing
130 the temperature until 41°C was reached and then gradually reducing it back to 37°C. Once the
131 slow wave frequency had returned to the original frequency and pattern, the temperature was
132 reduced to 27°C before being gradually increased back to 37°C. If the recording was
133 sufficiently stable, it was maintained at the lower end of the temperature range for an
134 extended period of time (up to 45 min) before being increased again to 37°C. The total
135 recording time therefore varied with each horse, but typically lasting 90-120 min. The lower
136 end of the temperature range was chosen in order to simulate the cooling of intestine that may
137 occur during extra-abdominal exposure at surgery and which has been reported
138 experimentally [16] whilst the upper temperature limit was chosen in order to simulate severe
139 pyrexia.

140

141 The slow wave and temperature data were recorded in parallel and stored using an acquisition
142 system (Power Lab 8SP)^c interfaced to a desktop computer. The software package LabChart
143 Pro^c was used to analyse the frequency, duration of individual slow waves as well as
144 amplitude and RMP of the waveforms. The duration of the diastolic pause between slow
145 waves was arithmetically derived by subtracting the mean slow wave duration/min from the
146 mean slow wave cycle duration/min (i.e. from the start of one slow wave cycle to the start of
147 the next) for a given temperature.

148

The relationship between temperature changes and slow wave frequency, duration and interval, amplitude and maximum RMP variables was assessed using regression models including a baseline for each case and an autocorrelation component. The association between temperature and variables of interest were considered as both linear (absolute change in the variable per unit change in temperature) and logarithmic (proportional change in the variable per unit change in temperature). A linear regression analysis was therefore used to provide estimates of the absolute impact of temperature, and logarithmic regression analysis to estimate Q_{10} coefficient for comparison with other physiological effects of temperature.

The Q_{10} coefficient for each variable was calculated using the following formula: $\left[\frac{R_2}{R_1}\right]^{\frac{10}{(T_2-T_1)}}$ [18] where R and T represent the variable and temperatures at each of the 2 measurements. Q_{10} for each variable was estimated from the data using the temperature coefficient ($\hat{\beta}_{temp}$) from a regression model of Log(variable) on temperature. The estimated coefficient for temperature from this model describes the change in Log(variable) per unit change in temperature, hence Q_{10} is estimated as the antilog of $10 \times \hat{\beta}_{temp}$. Q_{10} coefficients were estimated using data over a temperature range of 26.5°C to 37.5°C. The critical p value for statistical significance was set at 0.05. Reported p-values are for linear regression models of variables on temperature. Where logarithmic models give a different conclusion regarding statistical significance, corresponding p-values are also reported.

Results

Electrical activity consistent with slow waves was recorded from the longitudinal smooth muscle layer of the ileum in all 9 horses. This activity was characterised by a fast depolarising upstroke, a variable plateau phase and a slower repolarising phase returning to the RMP (Fig 1). The addition of TTX (1 μ M) to the superfusion fluid in 2 horses had no effect on the ongoing electrical activity indicating that this activity was non-neuronal in origin.

In total, 134 impalements were made in the 9 tissue sections over the temperature range described. The slow wave frequency, duration, interval, amplitude as well as RMP were assessed as part of this. The data for the mean, median and range of these parameters at 27°C, 37°C and 41°C are described in Table 1.

In all 9 horses slow wave frequency was approximately linearly-related to the temperature over the range studied increasing by 0.5 cycles/min for each one degree increase in temperature ($p < 0.001$) ($R^2 = 0.95$) (Fig 2). The initial slow wave frequency resumed when the temperature was returned to 37°C. However, the time it took for the slow wave frequency to return to the value it had been at the onset of the recording appeared subjectively to be directly related to the period for which the temperature had been changed, although this time period was not statistically evaluated due to variations in recording times. Similarly, although the slow wave frequency progressively increased in parallel with the temperature, subjectively, these rates were often not capable of being sustained for more than a few minutes when at the top end of the temperature range before starting to drop again.

Slow wave duration and morphology also changed as the temperature was altered. This was particularly evident at lower temperatures when both the plateau phase as well as the repolarisation phase of the slow waves visually appeared to become markedly prolonged (Fig

1). Using the described regression models, the duration of slow waves became significantly longer at lower temperatures ($p < 0.001$, $R^2 = 0.89$) as did the interval between these ($p < 0.001$, $R^2 = 0.75$, Fig 2).

There was no significant association between RMP (maximum) and temperature ($p = 0.1$, $R^2 = 0.52$). There was a statistically significant association between slow wave amplitude and temperature using a logarithmic (proportional effect) model ($p = 0.05$, $R^2 = 0.53$), however this effect was not significant for a linear (additive effect) model ($p = 0.08$) ($R^2 = 0.50$) and was dependent on one case that displayed a strong association between temperature and slow wave amplitude. The data for both these parameters are also displayed in Fig 2.

The mean Q_{10} coefficient value for slow wave frequency between 26.5°C and 37.5°C was 2.2 (95% confidence interval 2.2-2.3) i.e. the frequency approximately doubled with a 10°C increase in temperature. Similarly, the mean Q_{10} coefficient value for slow wave duration over these temperatures was 0.52 (95% confidence interval 0.52-0.53) i.e. the duration approximately halved with a 10°C increase in temperature. The mean Q_{10} coefficient value for slow wave interval over these temperatures was 0.32 (95% confidence interval 0.32-0.33) i.e. significantly shortened by a factor of 3 with a 10°C increase in temperature. All these findings indicate that the generation of slow waves involve temperature dependent physiological processes.

Discussion

This study has demonstrated that *in vitro* slow wave frequency in the equine ileum is highly temperature sensitive and approximately linearly-related to the temperature over the range

222 studied. The finding is in agreement with previous studies in both the rabbit and mouse small
223 intestine [9-11] as well as in the stomach of various species including humans, dogs and
224 guinea pigs [25-27]. It has been proposed that an altered rate of cellular metabolism occur at
225 different temperatures which may also affect the mechanisms involved in generating slow
226 wave activity [11, 26].

227
228 The pacemaker current generated by ICC is thought to be initiated by Ca^{2+} release via IP_3
229 receptors on the endoplasmic reticulum [28]. Subsequent events are still controversial,
230 although it has been proposed that this stimulates Ca^{2+} uptake into adjacent mitochondria
231 which transiently reduces the Ca^{2+} concentration in the space close to the plasma membrane
232 thereby increasing the open probability of a non-selective cation channel conductance [29].
233 However, this theory has recently been challenged and a Ca^{2+} activated chloride channel has
234 been proposed to be the initiating current [30-31]. Regardless of the types of ion channel
235 involved, this conductance creates small inward-directed currents which activate T-type Ca^{2+}
236 channels resulting in the upstroke depolarisation of the slow wave [32-33]. These initial small
237 currents continue to summate into so-called unitary potentials which provide the current for
238 the subsequent plateau phase [34-35]. It seems likely that the repolarisation of the slow waves
239 occurs by inactivation of these inward currents and activation of outward K^+ currents toward
240 the end of the plateau phase [36]. The generated slow wave current is then conducted to
241 smooth muscle cells where they evoke similar membrane potential fluctuations [7] although
242 involving slightly different ionic mechanisms as described earlier.

243
244 In the current study, slow wave frequency was approximately halved at 27°C compared to the
245 frequency at 37°C . Increases in slow wave duration as well as in the diastolic pause between
246 the waveforms were important contributors to this finding as indicated by the calculated Q_{10}

values. Visual assessment of the slow wave patterns indicated that the plateau phase as well as the repolarisation phase were the components that became particularly prolonged at lower temperatures. Previous investigators who made similar observations suggested that the upstroke depolarisation, the plateau- and repolarisation phases of the slow wave cycle have different temperature sensitivities [9,11]. As several different ion channels are likely to be involved in the various phases of the slow wave cycle and because these channels may have differing temperature sensitivities [37], this may explain the recorded observations. Gating of ion channels requires conformational changes of channel proteins, and the rate of these conformational changes is temperature sensitive [38]. As the precise ionic mechanism for the pacemaker current has not been firmly established, it is difficult to speculate further as to the mechanisms involved. However, the findings in the current investigation are in agreement with previous studies in other species [9,11,18] indicating similar cellular processes between species.

The resting membrane potential was not significantly affected by altering the temperature. This is also in agreement with the findings in earlier studies [9,11]. Although the reason for this is still not clear, it is perhaps somewhat surprising as one would expect the maintenance of membrane potential to be a highly energy dependent process [12]. It is also unclear why one horse in the study showed a strong association between temperature and slow wave amplitude resulting in an overall statistically significant association. For all other horses there was no significant change in amplitude which is also consistent with findings in previous studies [9,11,25].

Interestingly, in an earlier *in vitro* study by El-Sharkawy and Daniel [9] contractile activity of rabbit jejunum was recorded in parallel with slow wave activity. It was noted that as the

272 temperature was lowered, the force of the contractile activity became gradually weaker and
273 was eventually lost at approximately 25°C. Activation of L-type Ca^{2+} channels is necessary
274 for depolarisation and contraction of smooth muscle cells [8]. Nakayama and Torihashii [38]
275 demonstrated that L-type Ca^{2+} channels are indeed also temperature sensitive which may
276 account for the observation by El-Sharkawy and Daniel [9]. This effect was not evaluated in
277 the current study as the L-type Ca^{2+} channel blocker nifedipine was added to the Krebs
278 solution order to facilitate stable intracellular impalements. However it is reasonable to
279 speculate that there may also be a similar effect in equine intestinal tissue.

280

281 The current study has demonstrated a negative effect on *in vitro* slow wave activity at 27°C
282 compared to 37°C. This may be of some clinical relevance during abdominal surgery where
283 often lengthy general anaesthesia may result in mild to moderate hypothermia of the horse
284 [15]. In humans, an association between hypothermia and development of POI and delayed
285 gastric emptying has been mentioned, although not critically assessed [39-40]. This finding
286 may also be relevant with respect to equine surgical patients developing POI. Bearing in mind
287 that resection and anastomosis is a risk factor for the development of POI [22], it is important
288 to acknowledge that the logistics of performing resection and anastomosis necessitate a longer
289 duration of anaesthesia and surgery, increased intestinal handling and also increased potential
290 for intestinal cooling; hence there could be a compounding of effects all exacerbating the
291 ileus.

292

293 Although not statistically assessed in the current study, it was the clear impression that the
294 longer the tissue was kept at 27°C, the longer it took for the initial slow wave frequency to
295 recover once the temperature was returned to 37°C. Again, this may be of relevance during
296 lengthy colic surgeries where significant cooling of intestinal segments during extra-

297 abdominal exposure may occur as has been reported in experimental animals [16-17]. This
298 cooling may also contribute to a generalised hypothermia of the animal during the
299 perioperative and immediate post-operative period [16]. Minimising extra-abdominal gut
300 exposure time, keeping exposed intestine covered, and keeping the temperature of the
301 intestinal and abdominal lavage fluids at body temperature during colic surgery may help
302 avoid this and therefore aid recovery of slow wave activity.

303
304 In contrast, slow wave frequency continued to increase at temperatures above 37°C. Although
305 unstable impalements prevented recording for prolonged periods at elevated temperatures, it
306 appeared that the tissue was unable to maintain increased slow wave frequencies for many
307 minutes before starting to drop again. Although the reason for this is not known, it is
308 reasonable to speculate that this may have been due to the cells being unable to maintain the
309 increased metabolic rate under these experimental conditions. This may also indicate that it is
310 not necessarily beneficial to artificially create elevated intestinal temperature during surgery.

311
312 In the current study mid-ileum rather than jejunum was evaluated. The reason for this is that
313 mid-ileum is easily identified and may therefore be harvested with minimal handling which is
314 important. Also, due to the increased thickness of its muscle layers technically it is easier to
315 prepare prior to recording compared to jejunum. As this group has previously established
316 standard methodologies of intracellular microelectrode recordings using equine ileum these
317 methods stand as a platform for comparison [13]. Although POI often involves jejunum, the
318 authors consider that it is acceptable to use the ileum as a model when demonstrating a basic
319 physiological response even if the inherent slow wave frequency is likely to be lower here
320 than in the jejunum [28].

321

Similarly, the reason for only evaluating the longitudinal muscle was based on previous experience and studies in this research group in which it has been reported that slow wave activity is more readily recorded here than in the circular muscle layer [13]. As it was aimed to keep the recording session as short as we could in order to try and avoid any possible effect that prolonged time may have on slow wave activity it was decided to only use the longitudinal muscle layer. In theory the slow wave, and therefore contractile, activity could be differently affected in the inner circular muscle layer. However, the authors considered this unlikely as it was felt that the influence of temperature on the basic metabolic process were likely to be the same in both muscle layers. The authors therefore think that only recording from the outer longitudinal muscle layer was likely to be representative for the full thickness intestinal segment.

In summary, the current study has demonstrated that *in vitro* slow wave activity in the equine ileum is highly temperature dependent. This may help improve our understanding of the basic physiological processes that are involved in generating and maintaining normal intestinal motility patterns in the horse and the factors that may influence these.

Authors' declaration of interests

No competing interests have been declared.

Ethical Animal Research

Research ethics committee approval was not required for this study using cadaver material. All samples were collected with owners' consent.

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Authorship

All authors have contributed to the study design, data analysis and interpretation, preparation of the manuscript and final approval of the manuscript. C. Fintl and G. Pearson have carried out the preliminary and actual experimental studies.

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Figure legends:

Fig 1: Slow wave recordings from the same horse at 27°C, 37°C and 41°C respectively illustrating the slow wave patterns consisting of a fast depolarising upstroke, a variable plateau phase and a slower repolarising phase returning to the resting membrane potential. The slow wave frequency is clearly slower at the lower temperature having both longer

durations as well as longer interval between cycles at 27°C compared to at 37°C. This is even more evident when compared to the recordings at 41°C.

Fig 2: Plot illustrating the effect of temperature on slow wave duration and cycle interval (s) as well as slow wave frequency (cycles/min) over a temperature range of 27-41°C. A strong linear relationship for all 3 parameters was evident. The effect of temperature on slow wave amplitude (mV) as well as resting membrane potential (RMP) (mV) are also displayed. These 2 parameters do not, however, display a similar relationship.

Tables

Table 1: Mean, [median] and range of slow wave parameters obtained from intracellular recordings obtained from longitudinal smooth muscle cells of the mid-ileum.

Temp (°C)	No. of impalements	Frequency (cycles/min)	Duration (s)	Interval (s)	Amplitude (mV)	RMP (mV)
27°C	16	4.53 [4.5]	8.61 [8.66]	5.27 [5.39]	3.17 [2.5]	-40.12 [37.5]
		4.0-5.57	7.05-9.63	3.67-7.1	1.3-10.0	20.7-70.4
37°C	25	10.08 [10]	4.38 [4.28]	1.63 [1.48]	2.96 [2.1]	-37.96 [36.9]
		9.0-11.5	3.75-4.97	1.7-1.99	1.2-3.7	27.1-51.5
41°C	10	11.56 [11.5]	3.64 [3.66]	1.49 [1.55]	2.9 [2.5]	-34.51 [34.11]
		11.0-12.37	3.38-4.01	1.03-1.78	1.1-5.5	20.6-53.0

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Figure 1: Slow wave recording from the same cell at 27°C, 37°C and 41°C respectively illustrating the slow wave patterns consisting of a fast depolarising upstroke, a variable plateau phase and a slower repolarising phase returning to the resting membrane potential. The slow wave frequency is clearly slower at the lower temperature and the slow waves have both longer durations as well as longer interval between cycles than at 37°C. This is even more evident when compared to the recordings at 41°C.

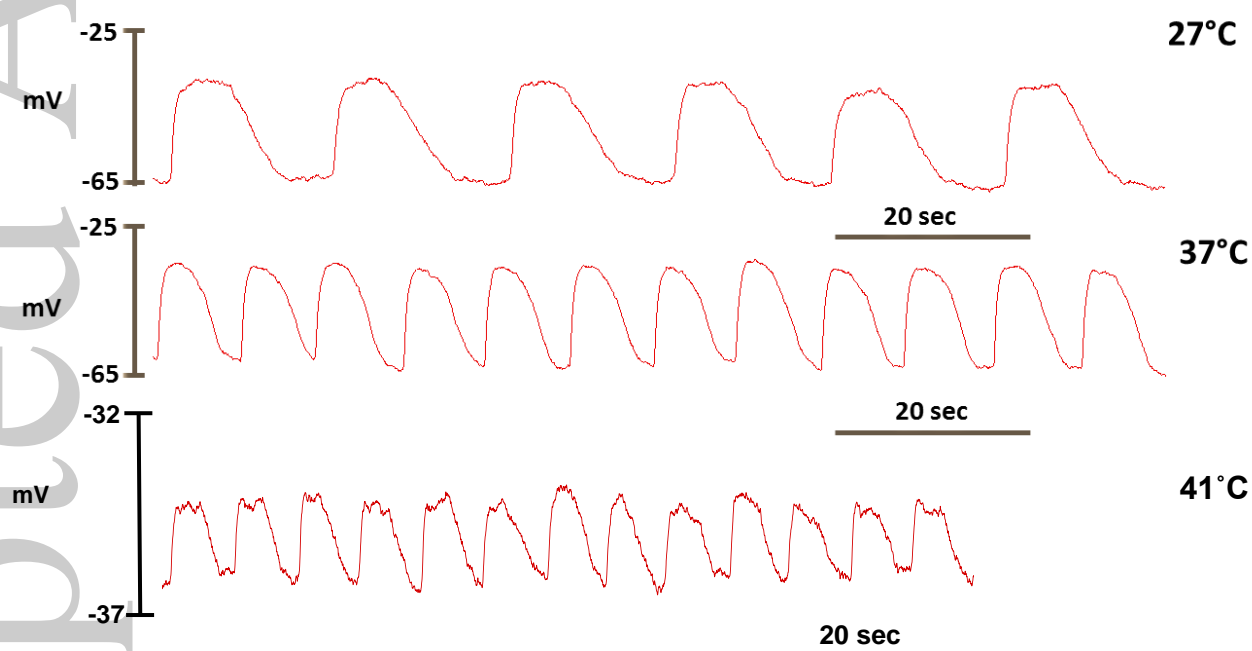


Figure 2: Plot illustrating the effect of temperature on slow wave duration and cycle interval (seconds) as well as slow wave frequency (cycles/minute) over a temperature range of 27-41°C. A strong linear relationship for all three parameters was evident. The effect of temperature on slow wave amplitude (mV) as well as resting membrane potential (RMP) (mV) are also displayed. These two parameters do not, however, display a similar relationship.

